THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 35, pp. 23842–23851, August 28, 2009

An in *Trans* Interaction at the Interface of the Helicase and Primase Domains of the Hexameric Gene 4 Protein of Bacteriophage T7 Modulates Their Activities*

Received for publication, May 27, 2009, and in revised form, June 30, 2009 Published, JBC Papers in Press, July 1, 2009, DOI 10.1074/jbc.M109.026104

Bin Zhu[1], **Seung-Joo Lee**[1], **and Charles C. Richardson**[2]

From the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

DNA helicase and primase are essential for DNA replication. The helicase unwinds the DNA to provide single-stranded templates for DNA polymerase. The primase catalyzes the synthesis of oligoribonucleotides for the initiation of lagging strand synthesis. The two activities reside in a single polypeptide encoded by gene 4 of bacteriophage T7. Their coexistence within the same polypeptide facilitates their coordination during DNA replication. One surface of helix E within the helicase domain is positioned to interact with the primase domain and the linker connecting the two domains within the functional hexamer. The interaction occurs in *trans* such that helix E interacts with the primase domain and the linker of the adjacent subunit. Most alterations of residues on the surface of helix E (Arg^{404}, Lys^{408}, Tyr^{411}, and Gly^{415}) eliminate the ability of the altered proteins to complement growth of T7 phage lacking gene 4. Both Tyr^{411} and Gly^{415} are important in oligomerization of the protein. Alterations G415V and K408A simultaneously influence helicase and primase activities in opposite manners that mimic events observed during coordinated DNA synthesis. The results suggest that Asp^{263} located in the linker of one subunit can interact with Tyr^{411}, Lys^{408}, or Arg^{404} in helix E of the adjacent subunit depending on the oligomerization state. Thus the switch in contacts between Asp^{263} and its three interacting residues in helix E of the adjacent subunit results in conformational changes that modulate helicase and primase activity.

At the replication fork DNA helicase unwinds the duplex DNA to expose single-stranded DNA for use as templates for the leading and lagging strand DNA polymerases (1). The 5′ to 3′ polymerization of nucleotides by the leading strand DNA polymerase proceeds in a continuous manner, whereas synthesis on the lagging strand occurs in a discontinuous manner, generating Okazaki fragments. The synthesis of each Okazaki fragment is initiated by the extension of an oligoribonucleotide that serves as a primer for the lagging strand DNA polymerase. These oligoribonucleotides are synthesized in a template-directed manner by DNA primase. For the two polymerases to communicate with each other, the lagging strand folds back on itself such that the lagging strand DNA polymerase becomes part of the replisome. This association of the two polymerases enables both strands to be synthesized in the same overall direction, and synthesis of both strands proceeds at identical rates. The folding of the lagging strand creates a replication loop of lagging strand DNA that contains the nascent Okazaki fragment and the ssDNA[3] extruded behind the helicase. Single-stranded DNA-binding protein binds to the exposed single-stranded DNA to remove secondary structure, but it also interacts with the other proteins of the replisome to assist in the coordination of DNA synthesis (2).

Among the several protein interactions within the replisome, the interaction of the helicase with the primase is one of the most critical (2–6). The association of the primase with the helicase places it in position to catalyze primer synthesis on the single-stranded DNA extruded by the moving helicase. In addition, the higher affinity of the helicase for single-stranded DNA serves to stabilize the primase on the lagging strand. Perhaps the most important is the ability of the primase to communicate with the helicase. During the rate-limiting step of primer synthesis, leading strand synthesis would be expected to outpace lagging strand synthesis. The association of primase with helicase provides a mechanism by which helicase movement can be coordinated with primer synthesis (7).

The gene 4 protein of bacteriophage T7 is unique in that it contains both helicase and primase activities within the same polypeptide chain (see Fig. 1*A*). Although separate genes encode other replicative helicases and primases, they nonetheless require a physical association to function properly (2, 5). The helicase activity resides in the C-terminal 295 residues, and the primase activity resides in the N-terminal 245 residues (8). A linker of 26 residues separates the helicase and primase domains. The linker plays a critical role in the oligomerization of gene 4 protein (9). The primase and the helicase domains have been purified separately and shown to exhibit their activities independently (9–11). However, the presence of each domain has striking effects on the activity of the other (2).

Like other members of the Family 4 helicases, the helicase domain of gene 4 protein functions as a hexamer (see Fig. 1*B*). Members of this family assemble on single-stranded DNA with the DNA passing through the central channel formed by the

* This work was supported, in whole or in part, by National Institutes of Health Grant GM54397 (to C. C. R.).

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-1864; Fax: 617-432-3362; E-mail: ccr@hms.harvard.edu.

[3] The abbreviations used are: ss, single-stranded; Ni-NTA, nickel-nitrilotriacetic acid; DTT, dithiothreitol.

oligomerization (4, 12). The nucleotide-binding site of the helicase is located at the subunit interface located between two RecA-like subdomains that bind dTTP, the preferred nucleotide for T7 helicase (13–17). The location of the nucleotide-binding site at the subunit interface provides multiple interactions of residues with the bound dTTP (18). These interactions assist in oligomerization, in binding to DNA, and in coupling the hydrolysis of dTTP to mechanical movement of the helicase (19–23).

The primase domain, residing in the N-terminal half of the gene 4 protein, is a member of the DnaG family of prokaryotic primases. Three structural features distinguish members of this family. An N-terminal zinc-binding domain plays a critical role in recognizing sites for primer synthesis in ssDNA. An RNA polymerase domain, linked to the zinc-binding domain by a flexible linker, contains the catalytic site where metal-dependent polymerization of nucleotides occurs. A C-terminal segment covalently attaches the primase to the helicase. In other primases of this family, this segment interacts with the cognate helicase. T7 primase, like the primases of phage T4 and *Escherichia coli*, recognizes a trinucleotide sequence (5). T7 primase recognizes the sequence 5′-GTC-3′, at which it catalyzes the template directed synthesis of a dinucleotide (pppAC); the 3′-cytosine is essential for recognition, although this "cryptic" nucleotide is not copied into the product (24). The dinucleotide is then extended by the primase, provided the proper nucleotides, T and G, are present in the template. Consequently, the predominant T7 primase recognition sites are 5′-GGGTC-3′, 5′-TGGTC-3′, and 5′-GTGTC-3′ (25, 26). Thus T7 primase catalyzes the synthesis of the tetraribonucleotides pppACCC, pppACCA, and pppACAC. The lagging strand DNA polymerase then extends these functional tetranucleotides.

The covalent linkage of primase and helicase in the gene 4 protein of bacteriophage T7 distinguishes it from most other replication systems where the association of the two proteins is dependent on a physical interaction of the two separate proteins. In bacteria such as *E. coli*, *Bacillus stearothermophilus*, and *Staphylococcus aureus*, this interaction is mediated through two structurally similar regions: the helicase-binding domain (p16 domain) located at the C terminus of the DnaG primase and the p17 domain of the DnaB helicase located at the N terminus of the protein (see Fig. 1*A*) (27–31). The association of DnaB with DnaG alters sequence recognition by DnaG and affects the length of primers synthesized (28, 31–33). Furthermore, cooperative binding of two or three DnaG monomers to the hexameric DnaB can halt translocation of DnaB on DNA (34). Such "association and dissociation" between the helicase and primase mediated by the p16 and p17 domains are believed to coordinate DNA synthesis by regulating the initiation of Okazaki fragment synthesis (6, 35, 36). Mutations in the p16 domain of DnaG can either affect the ability of the two proteins to form a complex, enhance the primase activity, or modulate the ATPase and/or helicase activities allosterically (31).

The covalent association of primase and helicase in the bacteriophage T7 system clearly provides several of the advantages derived from the physical association of the two proteins in other systems. The primase is positioned correctly for primer synthesis, and DNA binding is achieved via the helicase. Furthermore, communication between the two domains of the gene 4 protein is dramatically revealed by the cessation of helicase movement during primer synthesis (7). However, the covalent association of the two activities precludes regulation by dissociation as in the other replication systems. The frequency of primase recognition sites in the phage genome is considerably more than that required for the initiation of Okazaki fragments. Consequently, primase activity in the T7 replication system must be highly regulated to ensure the translocation of helicase and the almost constant length of Okazaki fragments (2).

T7 gene 4 protein is present in solution as a mixture of hexamers and heptamers (37), and the crystal structures of both oligomeric forms have been determined (see Fig. 1, *B* and *C*) (15, 38). In the heptameric structure an interaction of the helicase and primase domains occurs through helix E (see Fig. 1*C*). Located at the front of the helicase domain facing toward the primase domain, helix E is not only in proximity to the primase of the adjacent subunit but also in contact with the linker region connecting the two domains of the adjacent subunit. By this *trans*-packing interaction, the primase domain from one subunit is loosely stacked on the top of the helicase from the adjacent subunit (38) (see Fig. 1*C*). In the six-membered ring structure (15), the functional form of gene 4 protein, the primase domain is missing. However, the contact between helix E and the linker region from the adjacent subunit is present (see Fig. 1*B*). Some residues in the linker region have been identified previously as key factors involved in the conformational switch of helicase (39).

How does the primase domain of gene 4 protein communicate with the helicase domain? Although the two domains cannot dissociate into solution, a transient dissociation of the two domains is possible as a result of the flexible linker through which they are connected. Alternatively, primase activity or helicase activity may be conveyed to the other domain as a result of conformational changes in the protein at the interface between the two domains. In either instance the linker region and the interface between the two domains are certain to be critical for this communication. Helix E, although quite distant from the catalytic sites of either the helicase or primase, contacts both the primase domain and the linker. In the present study we have examined the role of helix E in the function of gene 4 by genetically altering several residues and examining the function of the altered proteins *in vivo* and *in vitro*.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were obtained from Integrated DNA Technology. Plasmid DNA purification kits and Ni-NTA resin were from Qiagen. Restriction endonucleases, Deep Vent® polymerase, and T4 DNA ligase were purchased from New England Biolabs. DNA-binding membranes were from Bio-Rad. Radiolabeled nucleotides were purchased from PerkinElmer Life Sciences. Agarose and β,γ-methylene dTTP were from USB Corp. Polyethyleneimine cellulose TLC plates were from EMD Chemicals.

Construction of Plasmids, Overproduction, and Purification of Gene 4 Protein—The coding region of T7 gene 4 was inserted into plasmid pET-28b (EMD Biosciences) between the NdeI

and HindIII sites. Site-directed mutations were introduced by a standard procedure including PCR amplification, restriction digestion, and ligation as described previously (40). The entire gene 4 coding region in the constructed plasmids was confirmed by DNA sequence analysis. The plasmids were transformed into *E. coli* BL21(DE3), and gene 4 proteins were overproduced as recombinant proteins containing a six-histidine tag at the N terminus. The bacteria were cultured until they reached an A_{600} of ~1, and gene 4 expression was induced with isopropyl β-D-thiogalactopyranoside at a final concentration of 1 mM at 37 °C for 3 h. The cells were harvested, resuspended in buffer (20 mM Tris-HCl, pH 7.5, and 0.15 M NaCl), and ruptured by three cycles of freeze-thaw in the presence of lysozyme. Clear lysate was collected by centrifugation and loaded onto a Ni-NTA affinity column (Qiagen) in the presence of 100 mM imidazole. Gene 4 protein bound to the Ni-NTA column was eluted using 500 mM imidazole. After dialysis to remove the imidazole, gene 4 protein was further purified using an ATP-agarose affinity column followed by DEAE anion exchange chromatography (40). All of the purified proteins were greater than 95% pure as determined by SDS-PAGE analysis and staining with Coomassie Blue.

Oligomerization Assay—The ability of gene 4 protein to oligomerize was determined by identifying oligomeric forms of the protein by nondenaturing polyacrylamide gel electrophoresis. The reaction (15 μl) contained 1.2 μg of gene 4 protein, 40 mM Tris-HCl, pH 7.5, 10 mM DTT, 10 mM $MgCl_2$, 50 mM potassium glutamate, 1 μM 25-mer oligonucleotide (5′-CGTAA TCTGC AGGCA TGGTC AATTT-3′), and 1 mM β,γ-methylene dTTP. For concentration-dependent oligomerization, a range of gene 4 protein (0.3–4.8 μg) was used. After incubation for 10 min at 37 °C, the reaction mixture was loaded onto a 10% nondenaturing polyacrylamide gel and run at 4 °C for 12 h in gel running buffer (25 mM Tris-HCl, pH 7.0, 10 mM $Mg(OAc)_2$, and 190 mM glycine). The proteins were visualized by staining with Coomassie Blue.

ssDNA Binding Assay—Binding of gene 4 protein to ssDNA was measured using a nitrocellulose filter binding procedure. The reaction (10 μl) containing 40 mM Tris-HCl, pH 7.5, 10 mM DTT, 10 mM $MgCl_2$, 50 mM potassium glutamate, 0.5 mM β,γ-methylene dTTP, and 2.5 nM of 5′-^{32}P-labeled DNA (5′-$GGGTCA_{10}$-3′) was incubated with various amounts of gene 4 protein at 37 °C for 10 min. The reaction mixture was loaded onto two layers of filters, a nitrocellulose membrane (0.45 μm) laid atop a Zeta-Probe® membrane fixed on a Dot microfiltration apparatus (Bio-Rad). The amounts of the protein-DNA complex bound to the nitrocellulose membrane and free DNA bound to the Zeta-Probe® membrane were measured using a Fuji BAS 1000 Bioimaging analyzer.

dTTP Hydrolysis Assay—DNA-dependent hydrolysis of dTTP by gene 4 protein was determined by incubating 0.25 mM [α-^{32}P]dTTP (0.1 μCi) in a 5-μl reaction volume with the indicated concentration of the protein in the presence of 40 mM Tris-HCl, pH 7.5, 10 mM DTT, 10 mM $MgCl_2$, 50 mM potassium glutamate, and 8 nM M13 ssDNA at 37 °C for 20 min. After termination of the reaction by the addition of EDTA to a final concentration of 25 mM, the reaction mixture was spotted onto a polyethyleneimine cellulose TLC plate. The TLC plate was developed with a solution containing 1 M formic acid and 0.8 M LiCl. The amount of [α-^{32}P]dTDP formed in the reaction was measured using a Fuji BAS 1000 Bioimaging analyzer.

DNA Unwinding Assay—A mini-replication fork substrate (see *inset* to Fig. 5*A*) used in the DNA unwinding assay was prepared by annealing a 5′-^{32}P-labeled 45-mer (5′-ATAAC TCTAT GCACA TTGAC CATGC TTCAG ATTCG TATTG TTACT-3′) to a 65-mer (5′-TTTTT TTTTT TTTTT TTTTT ATTCG TAATC CGACC TCGAG GCATG GTCAA TGTGC ATAGA GTTAT-3′) in 50 mM NaCl. The DNA substrate (100 nM) was incubated with the indicated amounts of gene 4 protein at 37 °C for 5 min in a buffer containing 40 mM Tris-HCl, pH 7.5, 10 mM DTT, 10 mM $MgCl_2$, 50 mM potassium glutamate, and 0.5 mM dTTP. After termination of the reaction by the addition of EDTA to a final concentration of 25 mM, the reaction mixture was loaded onto a 10% nondenaturing polyacrylamide gel. Radiolabeled oligonucleotides separated from the partial duplex substrate by the helicase were measured using a Fuji BAS 1000 Bioimaging analyzer.

Oligoribonucleotide Synthesis Assay—The ability of the gene 4 protein to catalyze template-directed oligoribonucleotide synthesis was determined by measuring the incorporation of [α-^{32}P]CMP into oligoribonucleotides using a synthetic DNA template containing a primase recognition site. The reaction (5 μl) included 40 mM Tris-HCl, pH 7.5, 10 mM DTT, 10 mM $MgCl_2$, 50 mM potassium glutamate, 0.1 mM each ATP and [α-^{32}P]CTP (0.1 μCi), 100 μM 6-mer DNA template (5′-**GGGTC**A-3′, a primase recognition site in bold) or 5 μM 60-mer DNA template (5′-TTTTT TTTTT TTTTT TTTTT ATTCG TAATC TGCAG GCA**TG GTC**AA TTTTT ATAGA GTTAT-3′, a primase recognition site in bold), and the indicated amount of gene 4 protein. When dTTP or β,γ-methylene dTTP was added, their final concentration was 0.5 mM. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 2 μl of sequencing dye, and 2 μl of each reaction was loaded onto a 25% denaturing polyacrylamide sequencing gel containing 3 M urea. After the gel was dried for autoradiography, the amount of the incorporation of CMP into the major product, the tetraribonucleotide (pppACCC or pppACCA), was determined using a Fuji BAS 1000 Bioimaging analyzer.

RESULTS

The crystal structures of the full-length gene 4 protein (38) reveals limited contact between the helicase and primase domains (Fig. 1, *B* and *C*). Nonetheless, helix E (residues 402–416) in the helicase domain of one subunit is in close proximity to the primase domain and to the linker (residues 246–271) connecting the two in the adjacent subunit. For example, Gly^{415} in helix E lies ~3 Å from Ala^{225} and Gly^{226} in the primase domain located on the adjacent subunit of the oligomeric gene 4 protein (Fig. 1*C*). Similarly, Lys^{408} in the same helix is close (~3 Å) to Asp^{263} in the linker region. However, Asp^{263} is closer to Tyr^{411} (~4 Å) than Lys^{408} (~7 Å), both located in helix E, in the hexameric structure of gene 4 protein where the entire primase domain is missing (Fig. 1*B*) (18). Helix E consists mainly of aliphatic residues, suggesting hydrophobic interaction with its interacting partner. Yet four charged residues (Asp^{403}, Arg^{404}, Lys^{408}, and Arg^{413}) in helix E raise the possibility of charge-

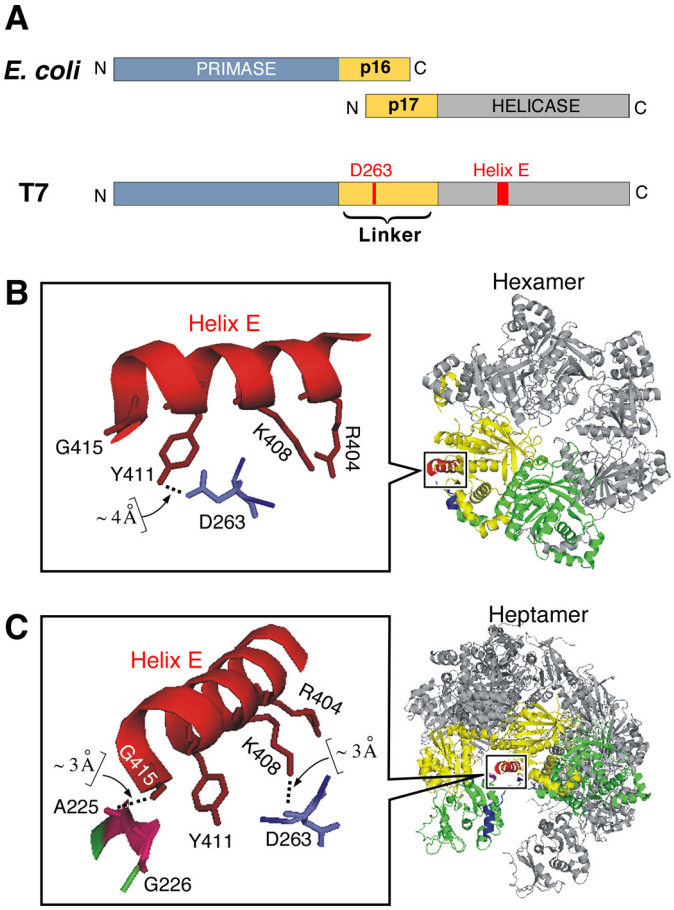

FIGURE 1. **Elements involved in the interaction between helicase and primase in *E. coli* and bacteriophage T7.** *A*, schematic presentation of helicase and primase together with the structural elements involved in their interaction. In *E. coli* the helicase and primase interact via contacts of the C-terminal p16 of the primase with the N-terminal p17 of the helicase. In bacteriophage T7 the two activities are found in a single polypeptide where the primase and helicase domains are covalently connected via a flexible linker. Helix E is located in the helicase domain. *B*, top view of the hexameric T7 helicase (*right panel*) (Protein Data Bank accession code 1E0J). *C*, side view of the heptameric gene 4 protein containing both the helicase and primase domains (*right panel*) (Protein Data Bank accession code 1Q57). In *B* and *C*, two adjacent subunits are shown in *green* and *yellow*, respectively. The linker region and residues Ala^{225}–Gly^{226} in the primase domain of the green subunit are shown in *blue* and *magenta*, respectively. Helix E in the helicase domain of the adjacent *yellow* subunit is shown in *red*. Residues potentially involved in the in *trans* interaction at the interface are indicated (*left panels*). In the heptameric structure (*C*), Gly^{415} in Helix E is potentially interacting with Ala^{225} and/or Gly^{226} from the primase domain of the adjacent subunit. Lys^{408} is close to Asp^{263} in the linker from the adjacent subunit. In the hexameric structure (*B*), because the primase domain and a portion of the linker region are missing in this structure, the counterpart of Gly^{415} is not present. Another obvious difference in this structure compared with that shown in *C* is that Asp^{263} in the linker of the heptamer is oriented toward Lys^{408}, whereas it is close to Tyr^{411} in the hexamer structure. Distances shown in *B* and *C* are in similar ranges regardless of locations of interfaces in both hexameric and heptameric gene 4 protein structures. Structures from the Protein Data Bank were analyzed using PyMOL (DeLano Scientific LLC).

mediated interactions. Interestingly, Gly^{415}, Lys^{408}, and Tyr^{411}, described above, are all located on the same side of helix E. To investigate the role of helix E in an interaction between the helicase and primase domains, we made single amino acid substitutions in helix E of the helicase domain and examined their effect on the function of the altered proteins. We have focused on the four residues (Arg^{404}, Lys^{408}, Tyr^{411}, and Gly^{415}) located on the same side of the helix, the side facing the primase domain. Among them Gly^{415}, Lys^{408}, and Tyr^{411} showed the proximity to residues from the adjacent subunit as described above. Arg^{404}, Lys^{408}, and Tyr^{411} were replaced with either nonpolar alanine or negatively charged aspartic acid to examine the effect of charge. Gly^{415} was replaced with an aliphatic residue such as valine or leucine to examine the effect of side chain size.

TABLE 1
The ability of the gene 4 protein variants to support the growth of T7Δ4 phage lacking gene 4 was tested as described under "Experimental Procedures"

Alteration in gene 4 protein	e.o.p.[a]
None (wild-type protein)	1
R404A	$<1 \times 10^{-6}$
R404D	$<1 \times 10^{-6}$
K408A	$<1 \times 10^{-6}$
K408D	$<1 \times 10^{-6}$
Y411A	$<1 \times 10^{-6}$
Y411D	$<1 \times 10^{-6}$
G415A	0.2
G415V	0.4
G415L	$<1 \times 10^{-6}$
G415W	$<1 \times 10^{-6}$
D403R	0.05
S414A	0.5
S414D	0.5
R413A	0.3
R413D	$<1 \times 10^{-6}$

[a] e.o.p. indicates the efficiency of plating relative to wild-type gene 4 protein.

In Vivo Complementation of Gene 4 Deletion Phage—The effect of alteration of residues in helix E on the *in vivo* function of gene 4 protein was determined by examining the ability of the altered gene 4 proteins to complement T7 phage lacking gene 4 (T7Δ4). In this assay, functional gene 4 protein expressed from plasmid harbored in *E. coli* supports the growth of T7Δ4 and leads to plaque formation. The results summarized in Table 1 reveal that most alterations of residues in the same face of helix E (Arg^{404}, Lys^{408}, Tyr^{411}, and Gly^{415}) eliminate the ability of the altered gene 4 proteins to support T7Δ4 growth. The only exception observed occurred when Gly^{415} was replaced with small aliphatic residues such as alanine or valine; these substitutions allowed for T7Δ4 phage growth. However, substitution of large residues such as leucine or tryptophan for Gly^{415} results in an inability of the altered protein to complement, suggesting the importance of the side chain size at this position. Examination of residues at other sides of helix E reinforces the importance of the side facing the primase and linker domains; most mutations such as D403R, S414A, and S414D do not exhibit severe defects *in vivo* (Table 1). Although the R413D substitution gives rise to a gene 4 protein that cannot complement for T7Δ4 phage growth, a less drastic change (R413A) at the same position yields a protein that does complement for T7Δ4 growth (Table 1). None of alterations presented in Table 1 exhibit a dominant negative effect on the growth of wild-type T7 phage (data not shown).

Biochemical Activities of Altered Proteins—We have used gene 4 proteins containing a His tag at the N terminus in biochemical characterization of the altered proteins. The His-tagged wild-type gene 4 protein has helicase and primase activities comparable with that of the untagged wild-type gene 4 protein (data not shown). In addition, His-tagged wild-type

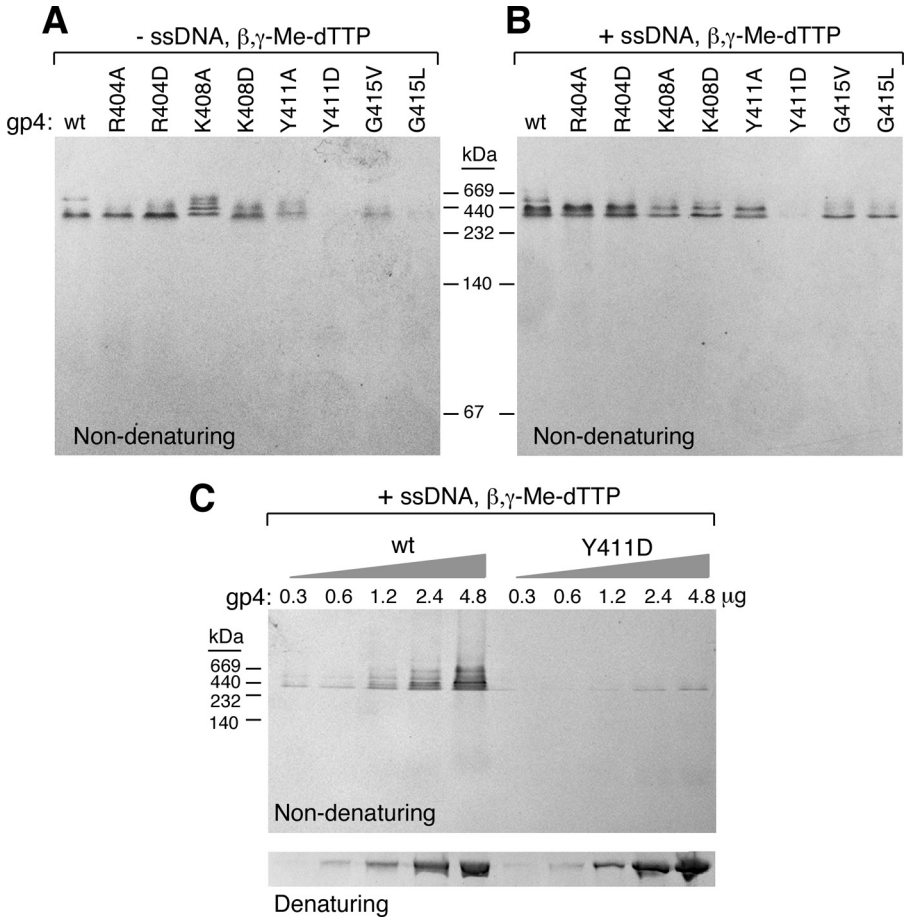

FIGURE 2. **Oligomerization of gene 4 protein.** *A*, oligomerization of gene 4 protein in the absence of cofactors. Gene 4 protein (1.2 μg) was incubated in the presence of 10 mM Mg^{2+} for 10 min at 37 °C and then analyzed on a nondenaturing polyacrylamide gel at 4 °C. *B*, oligomerization of gene 4 protein in the presence of ssDNA and β,γ-methylene dTTP. Gene 4 protein was analyzed by nondenaturing gel electrophoresis as in *A* except that 1 μM 25-mer ssDNA and 1 mM β,γ-methylene dTTP were present. *C*, concentration-dependent oligomerization of gene 4 protein. Increasing amounts (0.3, 0.6, 1.2, 2.4, and 4.8 μg) of either wild-type (*wt*) gene 4 protein or gp4-Y411D were incubated in the presence of ssDNA and β,γ-methylene dTTP. To ensure that equivalent amounts of proteins were used, the same amount of each protein was analyzed on a denaturing gel, and the resulting bands are shown at the *bottom* of *C*.

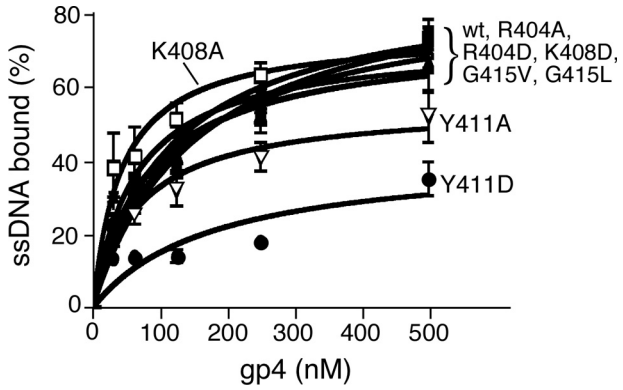

FIGURE 3. **ssDNA binding affinity of gene 4 protein.** Binding affinity of gene 4 protein to ssDNA was determined in the presence of β,γ-methylene dTTP using a filter binding assay as described under "Experimental Procedures." The indicated concentration of gene 4 protein was incubated with 2.5 nM 5′-radiolabeled 15-mer ssDNA at 37 °C for 10 min. The amount of gene 4 protein-bound DNA was measured and plotted against the protein concentration. Standard error was derived from at least three independent experiments. *wt*, wild type.

gene 4 protein supports the growth of T7Δ4 phage as well as the non-His-tagged version (data not shown). Thus we consider that the His tag does not affect our analysis.

Oligomerization—dTTP hydrolysis and DNA unwinding catalyzed by the helicase domain of gene 4 protein are dependent on its oligomerization because the NTP-binding site is located at the interface of adjacent subunits (18–21). The oligomerization is enhanced by the presence of ssDNA and nonhydrolyzable nucleotide such as β,γ-methylene dTTP. Oligomeric forms of gene 4 protein can be detected by electrophoresis on nondenaturing polyacrylamide (39). Most of the altered proteins oligomerize similar to wild-type gene 4 protein in the absence of β,γ-methylene dTTP and DNA (Fig. 2*A*). Two altered proteins, gp4-Y411D and gp4-G415L, are deficient in oligomerization. The ability of gp4-Y411D to oligomerize is not improved by the addition of ssDNA and β,γ-methylene dTTP, whereas gp4-G145L forms oligomers better in their presence (Fig. 2*B*). gp4-Y411D shows severe defects in oligomerization over a large range of concentration of the protein (Fig. 2*C*). In contrast, wild-type gene 4 protein progressively oligomerizes as the concentration is increased.

Binding to ssDNA—Gene 4 protein assembles as a hexamer on ssDNA with the DNA passing through the central core formed by oligomerization of the protein (4, 12). β,γ-Methylene dTTP, increases the affinity of the protein for ssDNA because it occupies the nucleotide-binding site but cannot support translocation of the protein on the bound DNA (41). We have measured the affinity of the altered proteins for ssDNA in the presence of β,γ-methylene dTTP using a filter binding assay (39, 41) (Fig. 3). None of the alterations affect DNA binding significantly except for alterations in Tyr^{411}. gp4-Y411D binds very poorly to ssDNA, an expected result because this altered protein is severely defective in oligomerization (Fig. 2*B*). gp4-Y411A binds less tightly to ssDNA at high protein concentrations as compared with wild-type gene 4 protein (Fig. 3).

Hydrolysis of dTTP—Gene 4 helicase translocates unidirectionally 5′ to 3′ on the ssDNA to which it is bound, using the energy of hydrolysis of dTTP (16). Upon encountering duplex DNA its continued movement unwinds the DNA. Hydrolysis of dTTP to dTDP and P_i occurs at the catalytic site quite distal to the binding of the ssDNA in the central core. The binding of dTTP, its hydrolysis, and release of the nucleotide products lead to conformational changes that eventually result in the DNA-binding loops in the central core moving the DNA

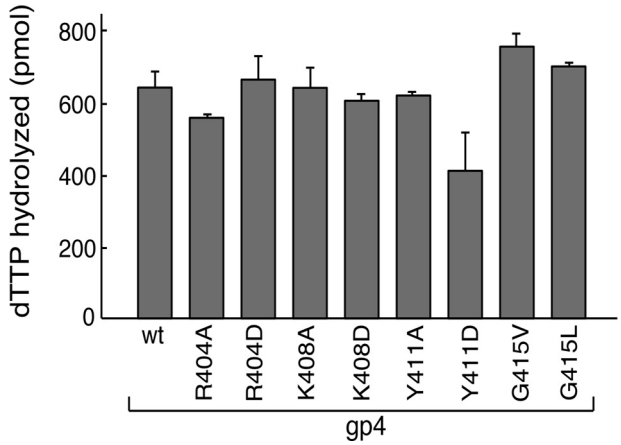

FIGURE 4. **dTTP hydrolysis activity of gene 4 protein.** The ssDNA-dependent hydrolysis of dTTP hydrolysis activity of gene 4 protein was measured as described under "Experimental Procedures." The indicated gene 4 protein (200 nM) was incubated with 0.25 mM [α-^{32}P]dTTP (0.1 μCi) in the presence of 8 nM M13 ssDNA at 37 °C for 20 min. The reaction products were separated by TLC, and dTTP hydrolysis was determined by measuring the amount of dTDP present in the reaction. The standard error was derived from at least three independent experiments. *wt*, wild type.

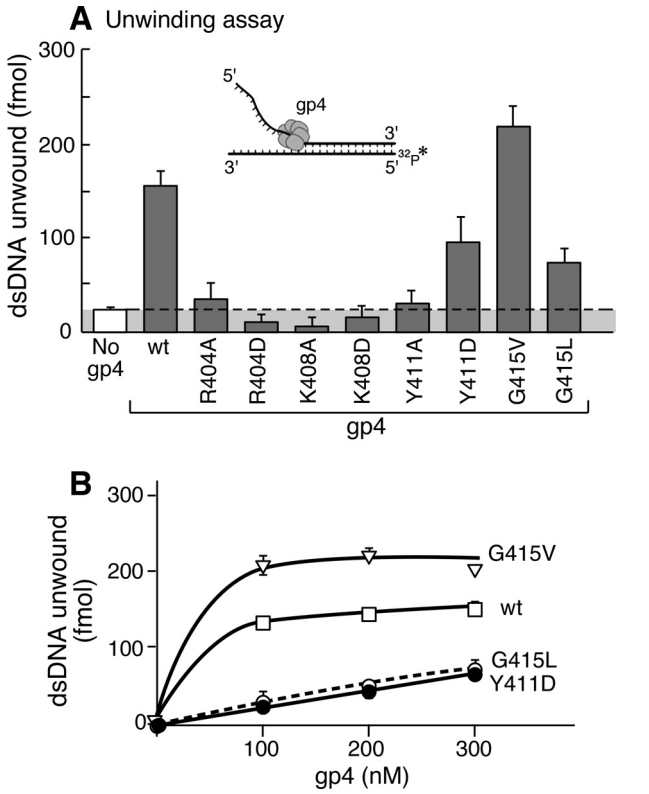

FIGURE 5. **DNA unwinding activity of gene 4 protein.** *A*, DNA unwinding activity of gene 4 protein was determined by measuring the amount of radiolabeled ssDNA displaced from a preformed replication fork (shown in *inset*) (500 fmol) by 200 nM of the indicated gene 4 protein as described under "Experimental Procedures." *B*, DNA unwinding activities of selected gene 4 proteins were examined at various concentrations of gene 4 protein. The amount of unwound substrate is plotted against the concentration of gene 4 protein. The standard error was derived from at least three independent experiments. *wt*, wild type.

through the protein (19, 21, 23, 41). Thus the hydrolysis of dTTP underlies all of the activities of the helicase domain of gene 4 protein. Each of the six subunits of the hexameric helicase hydrolyze dTTP randomly in the absence of ssDNA, albeit at a very slow rate. Upon binding to ssDNA, the rate of hydrolysis of dTTP by the subunit to which the DNA is bound increases ~50-fold (16, 23). In the presence of ssDNA, all altered proteins hydrolyze dTTP at approximately the same rate as wild-type gene 4 protein (Fig. 4). gp4-Y411D, defective in oligomerization (Fig. 2*B*), has lower activity than the wild-type protein.

DNA Unwinding—Unwinding of double-stranded DNA by gene 4 helicase can be measured using a DNA construct resembling a replication fork (Fig. 5*A*, *inset*). The assay measures the release of a radioactively labeled oligonucleotide partially annealed to a complementary ssDNA. The unwinding reaction is dependent on multiple properties of gene 4 protein: formation of a functional hexamer, binding to ssDNA, and efficient hydrolysis of dTTP to translocate on ssDNA. Most of the altered proteins retain these properties as described in the previous results. Nonetheless, we found that, with one exception, the altered gene 4 proteins are defective in the actual unwinding of duplex DNA (Fig. 5*A*). From the initial screening of the altered proteins for helicase activity, gp4-G415V appeared to be more active in unwinding DNA than the wild-type protein. Gp4-Y411D and gp4-G415L had ~50 and 30%, respectively, of the activity of wild-type gene 4 protein (Fig. 5*A*). The enhanced activity of gp4-G415V was confirmed by examining the unwinding of DNA as a function of gene 4 protein concentration (Fig. 5*B*). The defect in helicase activity of gp4-G415L and gp4-Y411D is most manifest at low protein concentrations where these enzymes have less than 10% of the activity of wild-type protein. Interesting, these two altered proteins were deficient in oligomerization (Fig. 2*A*), raising the possibility that the apparent unwinding at high protein concentration reflects a nonenzymatic interaction with DNA.

Oligoribonucleotide Synthesis—The primase domain of gene 4 protein resides in the N-terminal half of the protein. Unlike the helicase, the primase domain can catalyze the template-directed synthesis of oligoribonucleotides in the absence of oligomerization. Nonetheless, as discussed in the Introduction, the primase is affected by its association with the helicase. In particular, the tight binding of the hexameric helicase to ssDNA enhances the association of the primase with DNA, and the spatial arrangement of the two domains in the hexamer allows the zinc-binding domain of the primase on one subunit to functionally interact with the catalytic site of the primase on an adjacent subunit (42). Because helix E of the helicase domain faces the primase domain, we also examined the effect of alterations in helix E on primase activity.

First, we examined the ability of gene 4 protein to catalyze oligoribonucleotide synthesis on a 6-mer ssDNA template, 5′-GGGTCA-3′ containing the primase recognition site (5′-GGGTC-3′). Binding of the protein to such a short DNA is not affected by the helicase domain (43). A 2–3-fold reduction in oligoribonucleotide synthesis on the short template was observed with most of the altered proteins except for gp4-G415V, which has approximately the same activity as the wild-type gene 4 protein (Fig. 6*A*). However, when a 60-mer oligonucleotide containing the primase recognition sequence was used for the template-directed primer synthesis in the absence

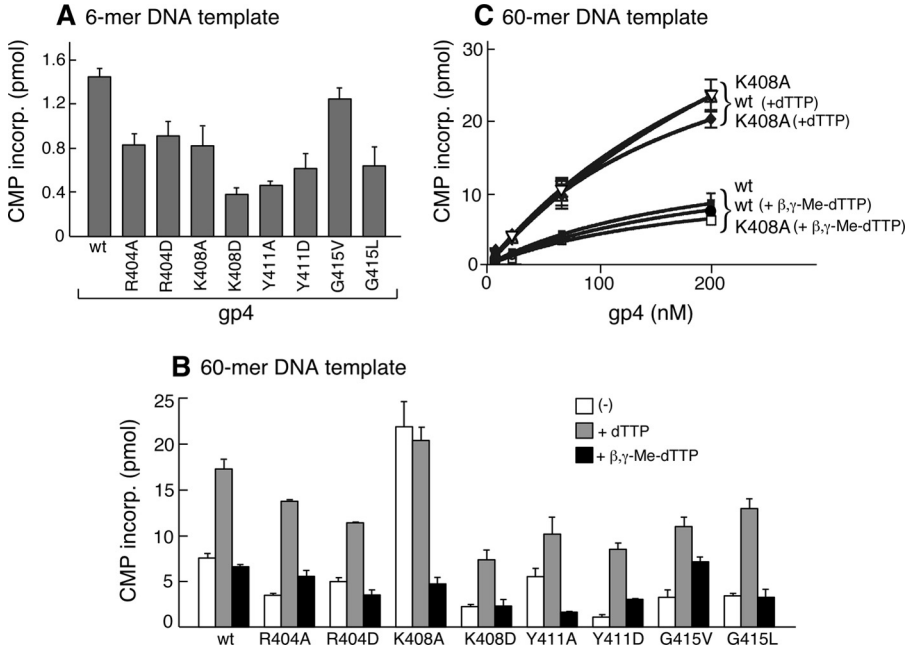

FIGURE 6. **Primase activity of gene 4 protein.** *A* and *B*, template-directed synthesis of oligoribonucleotide by gene 4 protein was determined by incubating 200 nM of the indicated gene 4 protein, 0.1 mM each of ATP and [α-^{32}P]CTP (0.1 μCi) with either a 6-mer (*A*) or a 60-mer (*B*) DNA template at 37 °C for 30 min. The 6-mer template contained the primase recognition sequence 5′-GGGTC-3′ and the 60-mer 5′-TGGTC-3′. Reaction products were analyzed on sequencing polyacrylamide gels, and incorporation of [α-^{32}P]CMP into the major product (tetraribonucleotide) was measured. Primer synthesis reactions using the 60-mer template were also performed in the absence or presence of 0.5 mM dTTP or β,γ-methylene dTTP. *C*, comparison of wild-type gene 4 protein with gp4-K408A for synthesis of oligoribonucleotides. The amount of radiolabeled CMP incorporated into tetraribonucleotide is plotted against the concentration of gene 4 protein. The standard error was derived from at least three independent experiments. *wt*, wild type.

of dTTP, gp4-K408A was 3-fold more active compared with wild-type protein (Fig. 6*B*). All of the other altered proteins were less active than wild-type gene 4 protein.

The addition of dTTP to the primase reaction catalyzed by the full-length gene 4 protein stimulates primase activity because dTTP enhances oligomerization and hence DNA binding and translocation on the DNA via the helicase domain (44). All of the proteins were stimulated by the addition of dTTP by approximately the same extent as wild-type gene 4 protein with the exception of gp4-K408A, whose already enhanced activity was not further stimulated (Fig. 6*B*). β,γ-Methylene dTTP had little effect on the synthesis of oligoribonucleotides by wild-type gene 4 protein, suggesting that the enhancement resulting from dTTP was a result of the translocation of the protein to the primase recognition site.

Perhaps the most interesting altered gene 4 protein with regard to primase activity is gp4-K408A whose elevated primase activity in the absence of dTTP is not stimulated by dTTP but is inhibited by β,γ-methylene dTTP. Concentration-dependent assays confirmed these results with gp4-K408A (Fig. 6*C*).

DISCUSSION

The functional form of the gene 4 helicase-primase of bacteriophage T7 in binding, translocation, and unwinding double-stranded DNA is a hexamer (18–20, 37). The crystal structure of the oligomeric helicase domain of gene 4 protein (15, 18) revealed two interfaces between adjacent subunits. In one interaction the N-terminal helix of one subunit packs against the neighbor subunit to form a bundle of four helices. In addition to this swapping of helices, several loops near the nucleotide-binding site interact with the nucleotide sandwiched between subunits or with residues in the adjacent subunit. The structure of the gene 4 protein containing both the helicase and catalytic sites of the primase domain (38) revealed that the primase domains are stacked onto the helicase domains of neighboring subunits and stabilized by *trans*-packing interaction of the linker connecting the primase and helicase domains. In the present study we show that interactions between the helicase domain of one subunit with the linker region and primase domain of the adjacent subunit are important in oligomerization as well as in modulating the activities of the gene 4 protein. In previous works the linker region (9) was shown to be indispensable for oligomerization. A small disturbance within this region such as changing residue Asp^{263} to Asn impedes the oligomerization of gene 4 protein (39). However, the structural explanation for these changes was lacking. By examining structures of the hexameric helicase domain and the heptameric helicase-primase (18, 38), we identified helix E on the outer surface of the helicase domain as a likely segment to interact with residues in the primase and linker domain.

An interesting aspect of the potential interaction is that it involves interactions of the helicase of one subunit with the primase and linker region of the adjacent subunit. Among the few residues involved in this interface, residues Asp^{263} in the linker and Tyr^{411} in helix E of the helicase (Figs. 1 and 7) are positioned for interaction in the crystal structure of the hexamer (18). Although Asp and Tyr are not generally considered to interact, interactions of the two have been implicated in a number of proteins (45). In some instances such an interaction plays an important role in maintaining the local conformation of a protein (46). Replacement of Tyr^{411} with aspartic acid severely hinders the oligomerization of gene 4 protein (Fig. 2). We had previously found that alterations of Asp^{263} also decrease oligomerization (39). These observations, taken together, suggest that an interaction of Asp^{263} with Tyr^{411} is important in the formation of gene 4 hexamers. One obvious role of this interaction is to stabilize the hexamer. However, the interaction between Tyr^{411} and Asp^{263} could regulate the switch from heptamer to hexamer, a transition that occurs upon loading of the hexamer onto DNA (37); Asp^{263} must change its orientation from that observed in the hexamer to establish the contact with Lys^{408} found in the heptamer (Fig. 1).

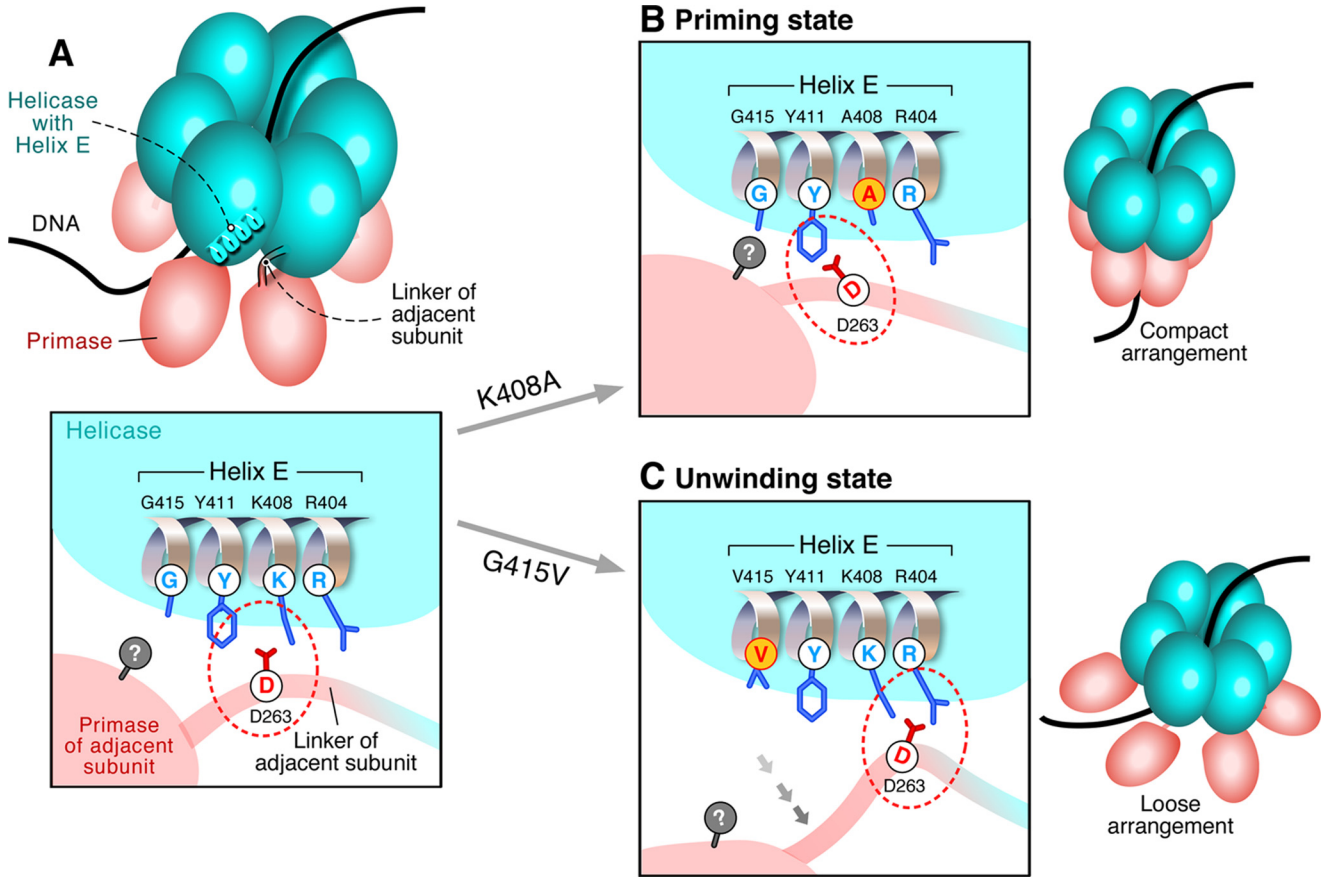

FIGURE 7. **Model for switching between helicase and primase function.** The potential interaction network at the interface between T7 helicase and primase domains in the hexamer functional unit (*A*) and the proposed switching among interacting residues in priming (*B*) and unwinding (*C*) states are shown. During DNA replication, the gene 4 hexamer exists in a relaxed conformation with the primase domains splayed out from the helicase domain (*C*). In this conformation residue Asp^{263} of one subunit contacts Lys^{408} and/or Arg^{404} in helix E of the adjacent subunit. Primase activity is silenced upon activation of the helicase. Increasing the size of the side chain size of Gly^{415} as in the Gly to Val construct increases the distance between the neighboring residues Tyr^{411} and Asp^{263} in the linker. This orientation favors the contacts between Asp^{263} and Lys^{408} and/or Arg^{404} and maintains the relaxed conformation of the gene 4 protein hexamer. gp4-G415V mimics the unwinding state with its elevated unwinding activity and diminished primer synthesis activity. At the appropriate time for primer synthesis, Asp^{263} switches its contact to Tyr^{411} in helix E of the adjacent subunit (*B*). In this conformation the primase domain is in close contact with the helicase domain, and the primase recognition sequence with primase activity is activated. At the same time the interface network can switch off helicase activity. Removal of the positive charge of Lys^{408} (Lys to Ala construct) favors the interaction between Tyr^{411} and Asp^{263}, because Lys^{408} and Tyr^{411} competes for contact with Asp^{263} (*A*). gp4-K408A mimics this priming state because this mutant possesses a high level of primer synthesis activity with a loss of unwinding activity.

Another residue involved in the oligomerization of gene 4 protein is Gly^{415}. Increasing the size of its side chain by replacement of the glycine with leucine leads to instability of the hexamer, whereas replacement with valine has no obvious defect. Thus Gly^{415} may stack on other residues to stabilize the hexamer. Unfortunately, no interacting residue can be predicted for Gly^{415} in the hexamer structure because the primase domain is not present (18). However, Ala^{225} or Gly^{226} have the potential to interact with Gly^{415} in the heptamer (38). The double mutant A225R/G226R does not exhibit any defect the *in vivo* complementation of T7Δ4 phage growth (data not shown).

T7 gene 4 protein is an essential component of the T7 replisome. It not only provides helicase and primase activities, essential activities for the leading and lagging stand synthesis, respectively, but it also interacts with T7 DNA polymerase and gene 2.5 ssDNA-binding protein (2, 47–49). All of these functions are dependent on the oligomerization of gene 4 protein for assembly at the replication fork. It is not surprising therefore that the defects in oligomerization seen in gp4-Y411D and gp4-G415L so strikingly affect T7 DNA replication *in vivo*.

The association of primase with DNA helicase has emerged as an important element in the coordination of leading and lagging strand DNA synthesis at the replication fork (7, 34, 50). For example, the synthesis of oligoribonucleotide primers by gene 4 protein on the lagging strand halts leading strand synthesis, presumably by halting helicase movement. In a bacterial system, the association and disassociation of helicase and primase as individual proteins differentiate the priming and unwinding state (6, 36). In the T7 replication system the association of the two activities has evolved into the covalent linkage of the two, an association that may expedite the process of DNA replication in phage-infected cells, albeit without all of the controls found in bacteria. Although the helicase and primase of T7 cannot be physically separated, mechanisms still exist for their communication with one another as mentioned above. One rationale for examining the interface between the helicase and primase domains of gene 4 protein was the obvious conclusion that any communication must pass through or involve this region. For example, even though the two

domains cannot part into solution, transient dissociations and reassociations of the two domains could still occur.

Our results suggest that contacts at the interface of helicase and primase can mediate this regulation. Single amino acid changes in helix E of this region result in changes in the primase and helicase activities that mimic events observed during coordinated DNA replication in the T7 system. For example, the K408A alteration enhances primase but inhibits helicase activity, simultaneous events observed during primer synthesis using single-molecule techniques (7). Consistently, gp4-K408A has a slightly higher affinity for ssDNA compared with wild-type gene 4 protein (Fig. 3). Interestingly, gp4-K408D differs from gp4-K408A in that it has significantly less primase activity, clearly showing that relatively minor alterations in helix E can regulate primase activity. Although gp4-K408A appears to be in a "priming mode," gp4-G415V is in an "unwinding" mode where helicase activity is activated and primase activity diminished. It is noteworthy that gp4-G415V does not oligomerize as well as wild-type protein, suggesting that a relatively loose conformation favors helicase activity. In contrast, the priming mode might arise from a more stable oligomer, as shown by gp4-K408A bound to DNA.

Residues Lys^{408}, Tyr^{411}, and Gly^{415} are on the same side of helix E, facing the primase domain. Residue Arg^{404}, also on this face, plays an essential role. Alterations of Arg^{404} lead to the loss of *in vivo* function as well as an inability to unwind DNA. The crystal structure of gene 4 protein does not reveal an interaction with another residue. However, more conformational change, for instance, induced by ssDNA binding could make it feasible for these residues to interact. In contrast, most alterations of residues located in helix E but not on the same surface as the four residues mentioned above do not lead to severe defects *in vivo*. From our data it appears that Asp^{263}, located in the linker of one subunit, interacts with Tyr^{411} in helix E in the hexamer and with Lys^{408} in helix E of the heptamer and with Arg^{404} in helix E. Thus the switch in contacts between Asp^{263} and the interacting residues in helix E of the adjacent subunit of the oligomeric gene 4 protein provides conformational changes to modulate helicase and primase activity.

A model to explain the effects of alterations in helix E on helicase and primase activity is presented in Fig. 7. During DNA replication the gene 4 hexamer exists in a relaxed conformation with the primase domains splayed out from the helicase domain (Fig. 7*C*). In this conformation residue Asp^{263} of one subunit contacts Lys^{408} and/or Arg^{404} in helix E of the adjacent subunit. Primase activity is silenced as the helicase domain unwinds DNA for the leading strand DNA polymerase. gp4-G415V mimics this conformational state. Gly^{415} contacts an unknown part of the primase domain. Increasing the size of the side chain of Gly^{415} might also increase the distance between the neighboring residues Tyr^{411} and Asp^{263} in the linker. Consequently, the G415V alteration favors contacts between Asp^{263} and Lys^{408} and/or Arg^{404} to maintain the relaxed conformation of the protein. However, larger size side chains of Gly^{415} result in instability of the hexamer, as shown by the deficiency of gp4-G415L in the oligomerization (Fig. 2*B*). At the appropriate time for primer synthesis, an unknown event triggers a switch such that Asp^{263} now contacts Tyr^{411} in helix E of the adjacent subunit (Fig. 7*B*). In this conformation the primase is activated with the primase domain in close contact with the helicase domain and the primase recognition sequence. gp4-K408A mimics this conformational state. Because Lys^{408} and Tyr^{411} might compete for the contact with Asp^{263} (Fig. 7*A*), as suggested by the hexameric and heptameric structures (Fig. 1, *B* and *C*), the removal of the positive charged Lys could favor the interaction between Tyr^{411} and Asp^{263}; by this interaction the primase can be pulled toward the helicase domain. This "programmed priming" could explain the frequency of initiation of Okazaki fragments where the number of priming events is far less than the number of primase recognition sites. When a primer is synthesized, the interface network can switch off helicase activity, a conformational state also mimicked by gp4-K408A. Single-molecule techniques have revealed that leading strand DNA synthesis mediated by the helicase and DNA polymerase temporarily halts during primer synthesis (7). Because the affinity of the primase for ssDNA and its recognition sequence is very weak, this "molecular brake" must arise from a cessation of helicase movement. Once the primer has been delivered to the polymerase, conformational changes at the interface similar to those observed with gp4-G415V reset the helicase activity for leading strand synthesis.

This switching mechanism once again illustrates the efficiency of replication that T7 has evolved to assure the rapid replication of its chromosome. The oligomeric state of the helicase domain is obviously important for binding to DNA and to translocation along the DNA where the DNA is essentially passed from subunit to subunit (21, 23). However, the oligomeric state also enables the individual subunits to communicate in *trans*, as is the case in the present study. This is not the first example of an in *trans* interaction with the T7 gene 4 protein. We have previously shown that the zinc-binding motif of the primase domain of one subunit can functionally interact with the RNA polymerase catalytic site of the primase domain located on the adjacent subunit (42, 51). These in *trans* interactions potentially regulate one another and thus play an indispensable regulatory role in coordinated DNA synthesis.

Acknowledgments—We thank Barak Akabayov for helpful discussion and Steven Moskowitz (Advanced Medical Graphics) for illustrations. We are grateful to Samir Hamdan for critical reading of manuscript.

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